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The purpose of this project is to develop and test the feasibility of a diagnostic blood test for breast cancer. Specifically, we are testing the feasibility of detecting breast cancer DNA shed into plasma (or serum) using methylation-specific PCR. In our studies to date, we have shown that large breast cancers shed sufficient quantities of DNA to allow detection using our proposed strategy. We have encountered several unexpected challenges to our expected application of this strategy for clinical application, however. First, many breast cancers do not have methylation of genes that we originally proposed for testing. We have successfully identified other genes that are frequently methylated in breast cancers, however, and will proceed in our project using these markers. A second problem that we have encountered is that the small serum or plasma sample size available does not frequently allow us to test multiple markers as proposed. We are addressing this problem by using a nested PCR approach that will allow us to test multiple markers from a single small sample. Additional ongoing studies will determine the limits of sensitivity for our assay in terms of tumor size and sample volume required.

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Introduction

As outlined in the grant application, we expected the majority of our work during the first year to be devoted to *Task 1*: to test the ability of MSP to detect breast cancer derived DNA in plasma of breast cancer patients. We have progressed in this area by 1) collecting suitably matched plasma (or serum) samples and breast cancer tissue samples, 2) screening tumor samples for methylation of specific genes, and 3) optimizing protocols for measuring methylation of DNA derived from plasma (or serum). We have also progressed as planned in our work outlined as *Task 2*. For this task, we have performed AP-PCR on several breast cancer samples and we have isolated, cloned, and sequenced one DNA segment that is commonly differentially methylated between matched normal and tumor samples. With new genome sequence information available through the Human Genome Project, we are initiating studies to characterize the corresponding gene.

Body

Accomplishments, problems, and proposed solutions are described with reference to the tasks outlined in the Statement of Work:

Task 1: To test the ability of MSP to detect breast cancer derived DNA in plasma of breast cancer patients (months 1 – 36)

- **Test matched serum/plasma and tissue samples for methylation of *e-cadherin*, *GST-pi*, *ER*, and *HIC-1* (months 1 – 24)**

  Accomplishments: As indicated in the Statement of Work, our major effort in the first year of funding was directed toward these proof-of-principal studies. The first level of achievement toward accomplishing this goal is the collection of matched plasma/serum and tumor tissue samples. During the first two year of funding, we have collected samples (matched plasma/serum and tissue) from 63 breast cancer patients.

  Serum/plasma samples are frequently limited in quantity and therefore it is essential to first screen tumor samples for methylation of specific genes before testing the serum/plasma samples. Using the methylation specific PCR reaction, we have tested 34 of the breast cancer samples and have found significant methylation of 8 genes, including the 4 genes originally proposed for use in the pilot studies. These additional genes were selected on the basis of our ongoing studies of gene methylation for markers of breast cancer (1, 2). The results for our tissue studies are summarized in the table below.

  Methylation of genes in breast cancer samples

	e-cad	*GST π*	*ER*	*HIC-1*	*Twist*	*RAR-B*	*HIN-1*
Number of samples tested	34	34	20	20	20	20	20
Number of samples with methylation	18	14	11	17	14	15	17

We have also conducted initial testing of 7 serum samples from patients with known breast cancer, using markers that we had previously determined to be

methylated in the respective tumor tissues. Unfortunately, our protocol detected methylated DNA in only 2 of the samples, both from patients with tumors greater than 3 cm in diameter and with lymph node metastases.

We believe that several factors are responsible for this limited sensitivity. First is that the genes tested have unmethylated as well as methylated alleles in the tumor tissue. This means that even if cancer-derived DNA is shed into plasma, only a portion of it is likely to be methylated. Our MSP reactions on tumor tissue were not quantitative and therefore we cannot ascertain the percentage of alleles in the tumor that were methylated.

A second factor that likely limits our sensitivity is the small amount of tumor-derived DNA that is likely to be present in the small volumes of plasma assayed. We have recently explored the use of nested PCR reactions after bisulfide modification to expand the amount of DNA available for assay. Finally, we must consider the possibility that small breast cancers do not shed significant amounts of DNA and our approach will inherently have a low sensitivity.

- **Test matched serum/plasma and tissue samples for methylation of novel sites identified by work of task 2 (months 18 – 36)**
  We have not yet initiated work defined by this task. As described above, we have expanded our number of marker genes through testing methylation of genes that are under-expressed in breast cancers. The novel gene studied in task 2 will be evaluated in the third year of the project.
- **Initiate additional studies of selected patient populations based on initial studies (months 24-36)**
  We have not yet initiated work defined by this task.

Task 2. To find novel aberrantly methylated sequences in breast cancers using methylation-specific arbitrarily primed PCR (months 6-30)

- **Perform AP-PCR on breast cancer samples (months 6-24)**
  Accomplishments: We have continued the AP-PCR work described as preliminary data for our grant application and we have repeatedly found a band suggestive of a sequence that is differentially methylated in breast cancers compared to normal tissues.
- **Isolate, clone, and sequence differentially methylated sequences from AP-PCR (months 12-30)**
  Accomplishments: The band described above has been isolated, cloned and sequenced. The sequence does not match any EST sequence deposited in the GenBank database. We have identified the sequence, however, in recently released data on the full human genome sequence. This should facilitate our characterization of this methylated sequence in the third year of this project.

It is notable that a number of genes have been described to be methylated in breast cancer over the past year. Among the most interesting is the HIN-1 gene, which is reportedly methylated in 74% of breast cancers (3). This progress will probably obviate the need to discover new markers through the AP-PCR approach.

Key Research Accomplishments

As described above, we have made progress in accordance with the schedule outlined in the Statement of Work. Although this progress will position us for testing the ability of the proposed strategy within the next year, we cannot yet report a definitive testing of our hypothesis at this time.

Reportable Outcomes

We have no reportable outcomes to date that specifically relate to a diagnostic blood test. Our work on identification of new markers of breast cancer has been incorporated in a study of breast duct lavage fluid for early detection of breast cancer (2).

Conclusions

Although we have experienced some unexpected difficulties with sensitivity of our assay, this project is still on track to test the hypotheses proposed in the origninal application. We are addressing some issues related to optimizing our methods for measuring DNA methylation in serum/plasma samples, and we expect to significantly improve our serum/plasma measurements over the nest year. One notable accomplishment is the characterization of new markers for breast cancer detection, which has contributed to a collaborative effort to detect breast cancer in duct lavage fluid. Although we have not yet characterized the gene corresponding to the novel aberrantly methylated sequence in breast cancer, the human genome sequence offers a new tool to complete this task.

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